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Purified recombinant hARD1 does not catalyse acetylation of Lys_{532} of HIF-1α fragments in vitro

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Abstract In humans, many responses to hypoxia including angiogenesis and erythropoiesis are mediated by the α/β-heterodimeric transcription factor hypoxia inducible factor (HIF). The stability and/or activity of human HIF-1α are modulated by post-translational modifications including prolyl and asparaginyl hydroxylation, phosphorylation, and reportedly by acetylation of the side-chain of Lys_{532} by ARD1 (arrest defective protein 1 homologue), an acetyltransferase. Using purified recombinant human ARD1 (hARD1) we did not observe ARD1-mediated *N*-acetylation of Lys_{532} using fragments of HIF-1α. However, recombinant hARD1 from *Escherichia coli* was produced with partial N-terminal acetylation and was observed to undergo slow self-mediated N-terminal acetylation. The observations are consistent with the other data indicating that hARD1, at least alone, does not acetylate HIF-1α, and with reports on the N-terminal acetyltransferase activity of a recently reported heterodimeric complex comprising hARD1 and *N*-acetyltransferase protein.

Keywords: Acetylation; hARD1; Hydroxylation; Hypoxia inducible factor; Oxygenase; Oxygen; Iron; Signalling; Transcription

1. Introduction

In many animals, cellular responses to reduced oxygen concentration (hypoxia) are mediated by an α/β-heterodimeric transcription factor, hypoxia inducible factor (HIF) [1]. Under hypoxic conditions, HIF binds to response elements linked to an array of genes related to the hypoxic response including, in humans, those associated with angiogenesis (vascular endothelial growth factor, VEGF) and erythropoiesis (erythropoietin, EPO). HIF-1β is a constitutively nuclear protein, but levels of HIF-1α are low under normal oxygen concentrations (normoxia) and rise in response to hypoxia [1].

Recent studies have identified mechanisms by which both the transcriptional activity and levels of HIF-1α are regulated by molecular oxygen. Post-translational *trans*-4-hydroxylation of either of two proline residues, that form part of consensus motifs located in the oxygen dependant degradation domain of human HIF-1α, is sufficient to target HIF-1α to the von Hippel–Lindau protein [2–4], which in turn recruits a ubiquitin ligase, that mediates proteasomal destruction. In humans, hydroxylation of an asparaginyl residue in the C-terminal transcriptional activation domain of HIF-1α ablates the interaction between HIF-1α and the p300 protein, thereby blocking transcription of HIF-1α-regulated genes [5]. Four hydroxylases have been identified that catalyse these post-translational modifications of human HIF-1α: three prolyl-hydroxylases (PHD1–3) [6,7] and an asparaginyl hydroxylase, factor inhibiting HIF (FIH) [8,9].

In addition to hydroxylation, HIF-1α polypeptides undergo other post-translational modifications and studies have identified sites of phosphorylation, ubiquitylation, and sumoylation [10–13]. Such modifications are of interest from the perspective of understanding the oxygen dependent regulation of HIF as they could enhance or reduce the transcriptional activity of HIF system even though they are not necessarily themselves direct redox transformations [14].

Jeong et al. have also reported that regulation of HIF-1α occurs via a mechanism involving acetylation of the side-chain of Lys_{532} of human HIF-1α, catalysed by hARD1 [15] [human analogue of yeast ARD1 protein; also known as ARD1 (from *ar*rest *d*efective protein *1*) or ARDH]. In yeast, ARD1 forms a heterodimer with NAT1 (*N-a*cetyl*t*ransferase protein); the resultant complex, (NatA) is anchored to the ribosome and catalyses N-terminal acetylation of nascent polypeptides [16]. The murine NAT complex (composed of mNAT and mARD) is important in neuronal development as it is highly expressed in areas of cell division but down regulated in areas of cell proliferation [17]. Formation of an analogous complex between hARD1 and hNAT1 in human cells has been observed using coimmunoprecipitation coupled to mass spectrometric analysis [18]. The reported observations of Jeong et al. with HIF-1α are potentially important as they imply that hARD1 is the first identified acetyltransferase to possess both an N-terminal acetyltransferase (in complex with *N*-acetyltransferase (human); NATH) and (by itself) lysyl side-chain acetyltransferase activity.

We have studied the acetyltransferase activity of purified recombinant hARD1 using fragments of HIF-1α and also a fragment of the peptide hormone ACTH (adrenocorticotropic hormone) as potential substrates. ACTH was chosen in the absence of a clearly defined hARD1 substrate as a representative known substrate of NatA which is acetylated on its N-terminal serine residue [18]. Although we did not observe acetylation of Lys_{532} of HIF-1α fragments or any acetylation of ACTH

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Abbreviations: HIF, hypoxia inducible factor; ARD1, arrest defective protein 1; NATH, *N*-acetyltransferase (human); EPO, erythropoietin; PHD, prolyl hydroxylase; FIH, factor inhibiting HIF; ACTH, adrenocorticotropic hormone; VEGF, vascular endothelial growth factor; HRE, hypoxic response element

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mediated by hARD1, we did observe that a slow self-acetylation reaction at the N-terminus of hARD1 takes place both on $(His)_6$-tagged hARD1 and the resulting $(His)_6$-tag-derived residues at the N-terminus after cleavage of the $(His)_6$-tag. N-Terminal acetylation of wild-type hARD1 was not observed, consistent with the predicted specificity of the ARD1-containing NatA complexes in yeast [19]. The results are consistent with studies that question the conclusions of Jeong et al. that hARD1 is involved in the regulation of HIF-1α activity [20,21].

2. Materials and methods

2.1. Cloning, overexpression and purification of recombinant hARD1

The DNA sequence encoding the *hARD1* protein was amplified by PCR from a construct donated by Prof. C. W. Pugh, (Wellcome Trust Centre for Human Genetics, Oxford University) as an *Eco*RI(*Nde*I/*Bam*HI)*Hin*dIII fragment using the primers 5′-CACCGGTGAATTCCATATGAACATCCGCAAT-3′ (sense) and 5′-TGGTGGAAGCTTGGATCCCTAGGAGGCTGAGTC-3′ (antisense), and ligated into the pMAL-c2x-1 vector (New England Biolabs). The *hARD1* gene was then excised as an *Nde*I/*Bam*HI fragment using the appropriate restriction enzymes and ligated into the pET28a(+) vector (Novagen). The pET28a/*hARD1* construct was transformed into BL21(DE3) *Escherichia coli* cells for protein expression, and the resulting $(His)_6$-hARD1 was purified with His-Bind resin (Novagen). When appropriate the $(His)_6$-tag was cleaved by overnight incubation with thrombin at 4 °C at a ratio of 2.5% (w/w) and further purification was achieved using a Superdex-S75 column (320 ml) equilibrated with 100 mM Tris–HCl, pH 7.5.

2.2. Cloning, overexpression and purification of HIF substrate fragments

A construct encoding the $HIF_{504–553}$ fragment in a modified version of the pGEX-6P1 vector was donated by Prof. C. W. Pugh, Wellcome Trust Centre for Human Genetics, Oxford University. The $hif_{401–603}$ fragment was cloned as a *Sac*II/*Asc*I fragment and ligated into a modified version of the pGEX-6P1 vector. Primers were 5′-TGGGGCCCGCGGATGGCCCCAGCCGCTGGAGACACA-3′ (sense) and 5′-GCTGGAGGCGCGCCATCCTGGAATCATGTAACTGTG-3′ (antisense). Constructs were transformed into BL21(DE3) *E. coli* cells and overexpressed as GST fusion proteins, and the resulting proteins were purified as previously reported using glutathione sepharose™ 4B resin (Amersham Biosciences) [9].

2.3. Analysis of acetyltransferase activity

Liquid chromatography–mass spectrometry (LC/MS) assays were performed using a Waters 600 high performance liquid chromatograph connected to a Micromass ZMD electrospray-quadrupole mass spectrometer operated in the positive ion mode. The HPLC was equipped with a Jupiter 5m C4 300A column (250 mm × 1.60 mm). The final concentration of substrate was 200 nM, the concentration of enzyme was 200 nM and the concentration of AcCoA varied between 200 nM and 2 μM. The protein was either assayed in a total sample volume of 50 μl, then precipitated with an equal volume of MeOH and 80 μl of the resulting sample was injected, or a total sample volume of 100 and 80 μl was injected with no precipitation step. [Controls were carried out under identical conditions but with hARD1 omitted and/or AcCoA omitted.] The solvent system used was: A: 95% H_2O/5% acetonitrile/0.1% HCOOH, B: 5% H_2O/95% acetonitrile/0.1% HCOOH, with the column equilibrated in 95% A before injection. The gradient was programmed as follows: 0–5 min 5% B isocratic; 5–35 min 5–95% B; 35–38 min 95% B isocratic; 38–40 min 95–5% B; 40–55 min 5% B isocratic.

For tryptic digestions, the protein of interest was isolated by SDS–PAGE, stained with Coomassie Blue stain, excised and destained using 100 μl of destain solution (50 mM ammonium bicarbonate in 50% acetonitrile) with agitation until the Coomassie stain was completely removed. The excised gel pieces were then treated with 80% aqueous acetonitrile for 20 min with no agitation before the acetonitrile was removed by pipette and the gel dehydrated by heating under reduced pressure for 30 min. Tryptic digestion was performed overnight using 50 μl of 20 ng/μl trypsin in 20 mM ammonium bicarbonate at 37 °C. The resulting supernatant was removed and peptides were extracted from the gel using 50 ml of 0.1% TFA in 50% acetonitrile (twice). These extracts were then combined and evaporated to dryness in a in vacuo before being reconstituted in 10 μl of 0.1% CF_3CO_2H immediately prior to use.

ESI/MS and ESI/MS/MS analyses were conducted using a Micromass (now Waters) Q-TOFmicro mass spectrometer. The capillary voltage was set to 3 kV and the sample and extractor cone voltages to 35 and 7.7 V, respectively. Nitrogen was used as the nebuliser and desolvation gases, and argon was employed for MS/MS collision induced dissociation. For LC/ESI-MS (and LC/ESI-MS/MS) the instrument was coupled to an Agilent 1100 capillary LC system equipped with a Phenomenex Jupiter C4 (150 mm × 0.5 mm) column using a gradient of water (0.1% formic acid) A and acetonitrile (0.1% formic acid) B as the mobile phase. The gradient was programmed as follows: 0–5 min 5% B isocratic; 5–30 min 5–43% B; 30–50 min 43–95% B.

For autoradiography analyses, the procedure of Ito [22] was followed. [1-^{14}C]-AcCoA (50 nCi) was incubated with enzyme (10 μg) and substrate (10 μg) and made up to a final volume of 10 μl with reaction buffer as described. The pH of the incubations was varied in one set of assays; subsequent self-acetylation studies were performed at pH 10. Controls were run with several proteins that contained an N-terminus consisting of a glycyl, serinyl or threoninyl residue, under the same conditions as those used for hARD1 and HIF substrates. The control proteins were FIH [9], $HIF_{530–698}$, open reading frames 2 [23], 15 and 16 from the clavulanic acid biosynthesis pathway (unpublished data), and phytanoyl coenzyme A hydroxylase [24], and were donated by D. Lancaster, A. Hardy, Dr. M. Caines and Dr. T. Searls, respectively (Chemistry Research Laboratory, Oxford University).

3. Results

3.1. Production of recombinant hARD1

The DNA sequence encoding hARD1 was prepared as an *Nde*I/*Bam*HI fragment and ligated into the pET28a(+) vector (as used by Jeong et al. [15]) in order to produce N-terminally $(His)_6$-tagged hARD1. The N-terminus of the resultant protein was GSSHHHHHHSSGLVPRGSHMNIR with the sequence of the hARD1 beginning from the 20th residue (underlined). ESI-MS analysis confirmed the presence of material of the predicted mass for $(His)_6$-hARD1 lacking the N-terminal methionine residue (predicted with N-terminal methionine 28 622 Da, without 28 492 Da; observed mass 28 496 Da). However, there was also a further peak apparent in the ESI-MS with a mass increase of 42 Da (Fig. 1, see below). Unoptimised cleavage trials with thrombin of the $(His)_6$-tag of the $(His)_6$-ARDH, at 4 °C, room temperature and 37 °C led to low yields of cleaved enzyme and so initial assays were performed using $(His)_6$-hARD1.

3.2. Evaluation of HIF fragments and ACTH as substrates for hARD1

The acetyltransferase activity of $(His)_6$-hARD1 was initially investigated in two ways: firstly, the $HIF_{504–553}$ fragment was incubated with $(His)_6$-hARD1 and AcCoA and studied by LC/MS; secondly, $HIF_{504–553}$ was treated with $(His)_6$-hARD1 and [1-^{14}C]-AcCoA and studied by [^{14}C]-autoradiography. During analysis by LC/MS, the unacetylated HIF fragment (observed as the $[M + 8H]^{8+}$ charge state at *m/z* 947, and the $[M + 7H]^{7+}$ charge state at *m/z* 1082) was clearly visible in the negative control (without enzyme) and also in the $(His)_6$-hARD1 containing incubation. There was no evidence of any acetylated $HIF_{504–553}$ in the enzyme-containing assay (Fig. 2). The $[M + 8H]^{8+}$ charge state of $HIF_{504–553}$ did contain

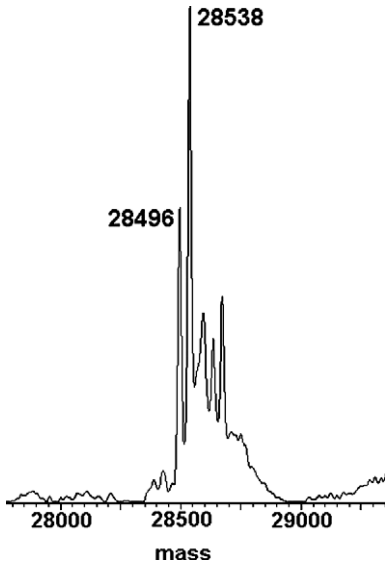

Fig. 1. ESI-MS analysis of $(His)_6$-tagged hARD1. The peak at 28 496 Da corresponds to the unacetylated hARD1 (predicted value 28 492 Da) whereas that at 28 538 Da corresponds to a mass increase of 42 Da and addition of an acetyl group. Other peaks correspond to sodium and potassium adducts.

low levels of peaks corresponding to a +16 Da shift, which would be consistent with methionine oxidation (the fragment contains one methionine residue). However, the presence of these minor peaks in both the assay mixture and the control incubation demonstrated that they were not introduced by a $(His)_6$-hARD1-mediated process. Autoradiographic analysis also did not lead to any evidence for $(His)_6$-hARD1-mediated acetylation of $HIF_{504–553}$ (Fig. 3A). Due to the failure of $HIF_{504–553}$ to act as a substrate, $HIF_{401–603}$ (reported as a substrate by Jeong et al.) was expressed as N-terminal GST fusion protein and used in partially purified form to investigate whether it acts as a substrate for hARD1 by [^{14}C]-autoradiography (Fig. 3B). Again no evidence for acetylation of the HIF fragment was observed.

A peptide corresponding to the first 24 N-terminal residues of ACTH (*a*drenocortico*t*ropic *h*ormone; predicted mass 2933 Da) was incubated with $(His)_6$-hARD1 and AcCoA to investigate the potential N-terminal acetyltransferase activity of hARD1. LC/MS analyses of the incubation revealed the peptide visible as the +3 and +4 charge states (at *m*/*z* 979 and 735, respectively) in its unacetylated form in both the assay incubation and in a no enzyme negative control (data not shown).

3.3. N-Terminal-acetylation of $(His)_6$-tag derived residues on hARD1

During studies investigating the acetyltransferase activity of $(His)_6$-hARD1 using HIF-1α fragments, a radioactive band corresponding to the position of hARD1 was observed by SDS–PAGE autoradiography, implying that a radioactive acetyl group had been transferred onto $(His)_6$-hARD1 itself (Fig. 3). As the N-terminal acetyltransferase activity of yeast NatA complex is reported to be selective for Ala, Gly, Ser and Thr residues [19], and the N-terminal residue of $(His)_6$-hARD1 was Gly (after N-terminal Met processing, the sequence of the first 9 residues of the protein was GSSHHHHHH) the possibility of $(His)_6$-hARD1 self-acetylation was investigated.

Directly after purification by Ni-affinity chromatography, the deconvoluted ESI-MS of $(His)_6$-hARD1 displayed a minor peak at 28 496 Da consistent with loss of the N-terminal Met residue of the $(His)_6$-tag with no modification. However, the major species present had a mass of 28 538 Da and corresponded to a mass shift of +42 Da consistent with partial acetylation (Fig. 1). Further minor peaks at higher mass were attributed to sodium and potassium adducts. Identification of the putative acetylation site was achieved using tryptic digestion and LC/MS analysis, which clearly identified the site of acetylation as the N-terminal peptide derived from the $(His)_6$-tag of the $(His)_6$-hARD1. No evidence for acetylation elsewhere on the $(His)_6$-hARD1 was accrued.

The N-terminal tryptic peptide had a predicted mass of 1767.8 Da (monoisotopic) and gave by LC/ESI-MS the +2 and +3 charge states (*m*/*z* 885.0 and 590.4, respectively). The acetylated form of the peptide had a predicted mass of 1809.8 Da and was visible as the +2 and +3 charge states (*m*/*z* 906.5 and 604.3, respectively). MS/MS analysis was performed on the ions at *m*/*z* 590.4 and 604.3 to confirm the sequence of the acetylated and unacetylated peptides and to assign the position of acetylation (Fig. 4).

To eliminate the possibility that the observed N-terminal acetylation site of $(His)_6$-hARD1 was not due to a self-acetylation but was occurring through *N*-acetylation by a bacterial *N*-acetyltransferase during expression, the $(His)_6$-tag was cleaved with thrombin to give hARD1 with an N-terminal sequence of GSHMNIRNARP, with the first three residues (underlined) being derived from the vector. The resulting GSH-hARD1 was incubated with [1-^{14}C]-AcCoA and controls were run with several proteins that contained a glycine residue at the N-terminus, under the same conditions as those used for hARD1 and HIF-1α substrates. The control proteins were FIH [9], $HIF_{530–698}$, open reading frames 2 [23], 15 and 16 from the clavulanic acid biosynthesis pathway, (unpublished data) and phytanoyl coenzyme A hydroxylase [24]. Autoradiographic analysis revealed that the band corresponding to GSH-hARD1 was [^{14}C]-labeled, suggesting that the acetylation was indeed self-mediated; a faint band corresponding to acetylated residual $(His)_6$-tagged hARD1 was also visible in assays containing enzyme with both [1-^{14}C]-AcCoA and ORFs 2, 15 and 16 (Fig. 5). The site of acetylation on the AcCoA-incubated hARD1 was identified as the N-terminus by incubating GSH-hARD1 with AcCoA, followed by tryptic digest/mass spectrometry as before. A +42 Da shift was observed on the peptide derived from the N-terminus of the GSH-hARD1. The identity of the N-terminal peptide and location of acetylation site was confirmed by MS/MS analysis. In the absence of AcCoA the N-terminal peptide showed no evidence of acetylation (data not shown). In none of the LC/MS analyses was any evidence accrued for side-chain acetylation of any of the 16 lysine residues of hARD1 (lysines 29, 51, 59, 78, 89, 105, 113, 136, 148, 165, 167, 179, 183, 198, 210, 225). The extent of hARD1 acetylation as a function of pH was studied in the range 5–11 using $(His)_6$-tagged hARD1; incubations at higher pHs showed a higher level of incorporation of [^{14}C]-label (Fig. 6), consistent with N-terminal amine acetylation.

After observation of the self-mediated N-terminal acetylation of unnatural residues on hARD1 the question of whether hARD1 may self-acetylate its natural N-terminus arose. Wild-

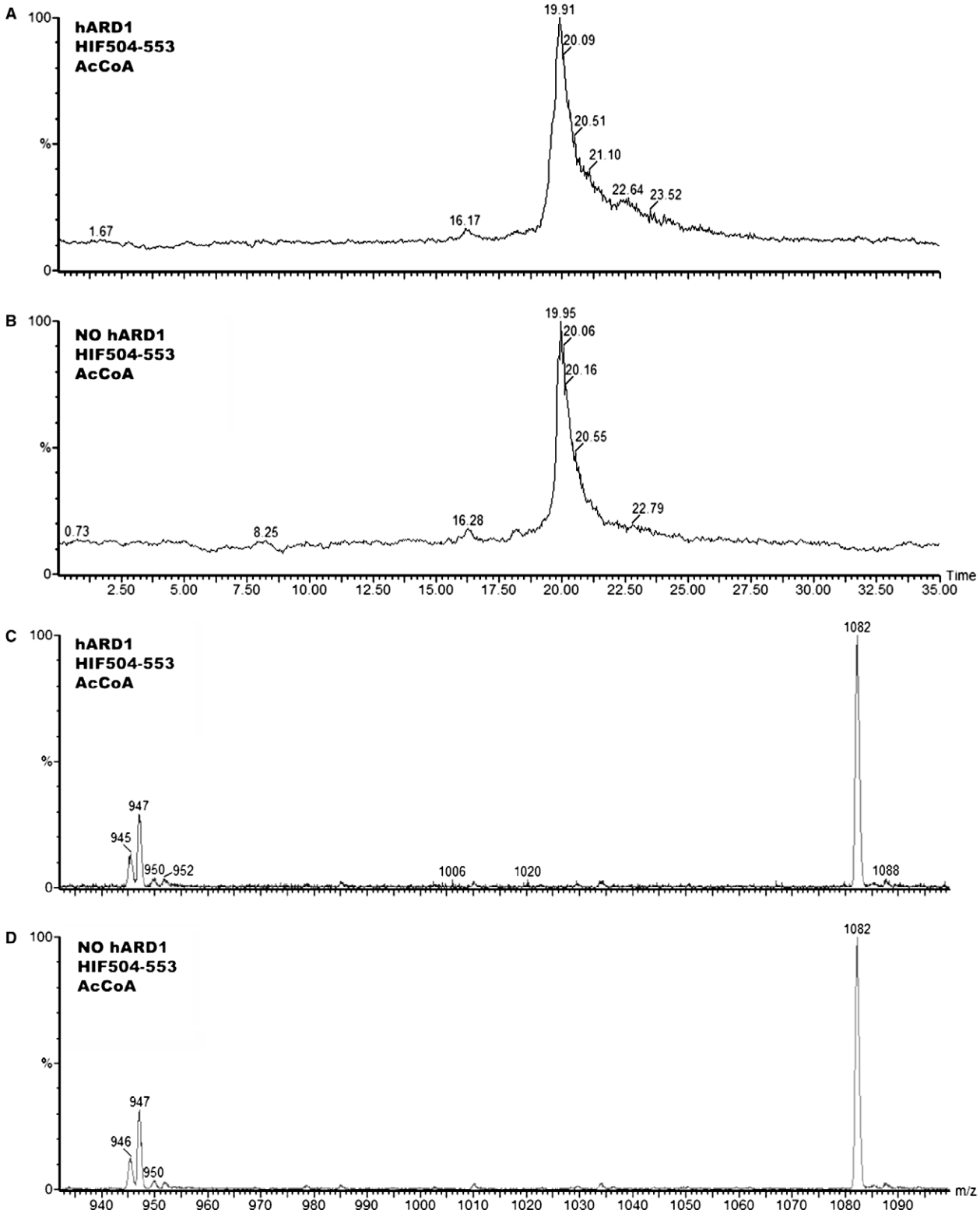

Fig. 2. LC/MS analyses of the incubation of $(His)_6$-tagged hARD1 with HIF-1$\alpha_{504–553}$ and AcCoA. (A) HPLC trace from incubation of $(His)_6$-tagged hARD1 with HIF-1$\alpha_{504–553}$ and AcCoA, (B) without $(His)_6$-tagged hARD1. MS data resulting from peaks in A and B, respectively, are shown in C and D. The peak at 1082 Da corresponds to the $[M + 7H]^{7+}$ charge state of HIF-1$\alpha_{504–553}$.

type hARD1 was produced in *E. coli* and the crude extract was incubated with either AcCoA, and analysed by SDS–PAGE, tryptic digestion and LC/MS, or [1-^{14}C]-AcCoA, and then autoradiographed. However, neither technique provided any evidence that the natural N-terminus of hARD1 was functioning as a substrate for self-acetylation (data not shown).

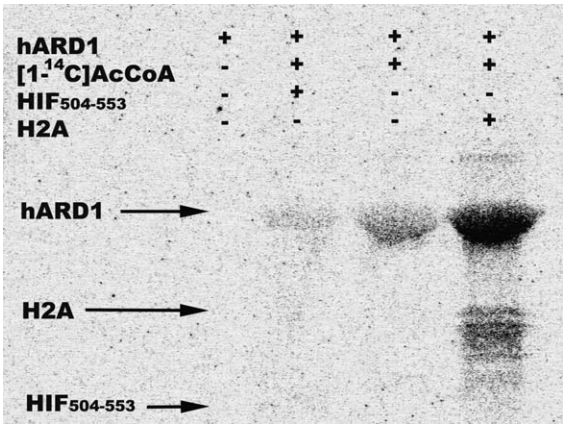

Fig. 3A. Autoradiograph from the incubations of $(His)_6$-tagged hARD1 with [1-^{14}C]-AcCoA and HIF-1$\alpha_{504–553}$. The arrowed band corresponds to acetylated $(His)_6$-tagged hARD1. H2A = Histone Type II A; there was no evidence for $(His)_6$-tagged hARD1-mediated histone acetylation as labeling of the H2A occurred in the absence of $(His)_6$-tagged ARD1.

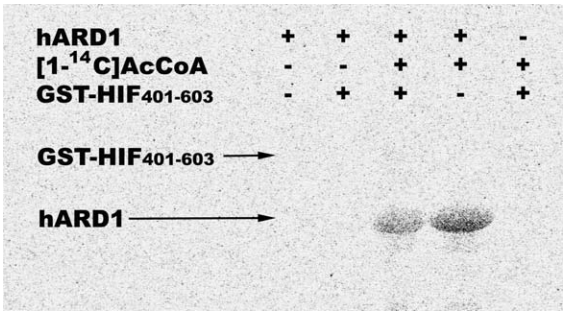

Fig. 3B. Autoradiograph from the incubations of $(His)_6$-tagged hARD1 with [1-^{14}C]-AcCoA and partially purified GST-tagged HIF-1$\alpha_{401–603}$. The arrowed band corresponds to acetylated $(His)_6$-tagged hARD1.

4. Discussion

Jeong et al. identified mARD1 as a protein that affected the stability of HIF-1α via a luciferase based reporter system utilizing pSV40promoter-EpoHRE-Luc (HRE = *h*ypoxic *r*esponse *e*lement) and mutated EpoHRE-Luc reporters [15]. Using an HRE-containing VEGF gene it was observed that an increase in VEGF mRNA production under hypoxia was abrogated in HT1080 cells overexpressing mARD1, implying hARD1 activated the HIF system. It was reported that recombinant mouse ARD1 (mARD1; 96% sequence identity with hARD1) produced using the pET28a vector catalysed the transfer of an acetyl group from AcCoA to the side-chain of a fragment of recombinant human HIF-1α corresponding to residues 401–603. The evidence for acetylation of the fragment was in part based upon [^{14}C]-autoradiography using [1–^{14}C]-AcCoA and Western blot analysis using anti-*N*-acetyl Lys antibodies. Western blot analyses of incubations with recombinant mARD1 and six lysine to arginine mutations of a HIF-1$\alpha_{401–603}$ fragment led to the initial assignment of Lys_{532} as the site of acetylation; support for the assignment came from isolation of acetylated HIF-1α by immunoprecipitation with anti-*N*-acetyl-Lys antibody and V8 protease-mediated digestion followed by analysis by MALDI-MS. A species with a mass of 1580.75 Da, corresponding to that predicted for the acetylated $HIF_{525–537}$ fragment (including with oxidation of a single methionine to a sulfoxide, and which contained no other lysine residues) was observed, leading to identification of the acetylation site as Lys_{532}. However, no MS/MS data was reported in this study.

Recently it has been reported that hARD1 does not affect the stability of HIF-1α and that hARD1 is not induced by hypoxia [20,21]. Fisher et al. used a retroviral vector to deliver hARD1 shRNA into HepG2 cells to knock down hARD1 message by >80%, and observed a resultant decrease in VEGF and EPO levels, in accordance with the earlier results of Jeong et al. However, they did not notice a decrease in the levels of HIF-1α protein, implying that knockout of hARD1 message was decreasing VEGF and EPO protein levels by a non-HIF-1α-mediated process. Bilton et al. also studied the effect of hARD1 on HIF-1α and -2α levels in HeLa and HT1080 cells by both overexpressing and silencing hARD1 [21]. When hARD1 was overexpressed as a tagged fusion protein in HeLa cells in both normoxia and hypoxia for 4 and 8 h no difference in HIF-1α levels between the control and the hARD1 overexpressing cells was observed. It was also observed that when hARD1 was silenced, there was no resultant stabilization in HIF-1α at the protein level. This observation contrasts with the fact that silencing of PHD2, known to mediate hydroxylation-dependent destruction of HIF-1α, strongly increases HIF-1α stability [21,25].

We investigated the acetyltransferase activity of highly purified hARD1 using the HIF-1$\alpha_{504–553}$ fragment via LC/MS and [^{14}C]-autoradiography employing [1-^{14}C]-AcCoA. We did not observe either a mass shift consistent with acetylation of the putative HIF-1α substrate, nor incorporation of any [^{14}C] into the HIF fragment. After this we used a larger fragment of HIF-1α, corresponding to residues 401–603 and containing the whole of the C-terminal oxygen dependent degradation domain, in an attempt to detect any acetylation using [^{14}C]-autoradiography, but we were not able to confirm the results of Jeong et al [15]. Thus, with respect to the HIF system our results imply that hARD1 does not, at least solely, catalyse acetylation of the amino side-chain of Lys_{532} of HIF-1α.

During [^{14}C]-autoradiography studies using $(His)_6$-tagged hARD1 a radioactive band corresponding to hARD1 was observed, which was consistent with incorporation of a [^{14}C]-labeled acetyl group. ESI-MS analyses also revealed that recombinant hARD1, with an unnatural N-terminus, produced in *E. coli* had undergone partial acetylation. Although it cannot be certain that all the acetylation of hARD1 occurring within *E. coli* is autocatalytic, subsequent experiments using LC/MS and tryptic digestion with LC/MS/MS demonstrated that recombinant hARD1 can catalyse slow self-acetylation at its N-terminus, but not, under the assay conditions, at the side-chains of any of the 16 lysine residues that it contains. The reaction was found to occur most efficiently at high pH consistent with a process involving solvent-mediated deprotonation of the N-terminal amino group. Acetylation at the N-terminus was observed for two different sequences [GSSHH- for $(His)_6$-tagged and GSHMN- for thrombin-cleaved hARD1] but not for the natural N-terminus of hARD1 (MNIRNARPED). These observations are consistent with the known selectivity of the yeast NatA complex, which can accept N-terminal serinyl-, glycyl-, alaninyl- and threoninyl-

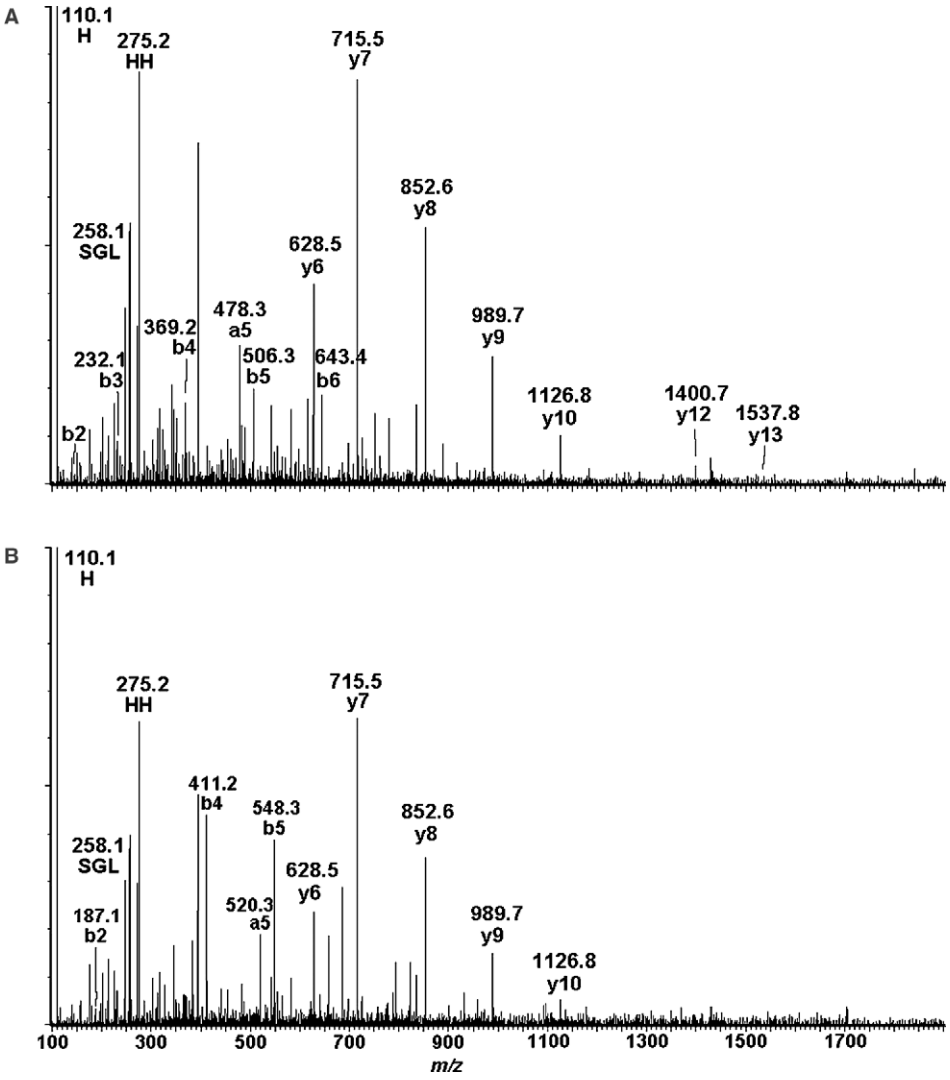

Fig. 4. MS/MS spectra of the $(His)_6$-tagged hARD1 N-terminal tryptic peptide, GSSHHHHHHSSGLVPR, (A) in the absence of AcCoA ($[M + 3H]^{3+} = 590.4$) and (B) in the presence of AcCoA ($[M + 3H]^{3+} = 604.3$). The presence of a b-ion series from b_2 to b_6 in both spectra formally assigns the site of acetylation to the first two N-terminal amino acid residues.

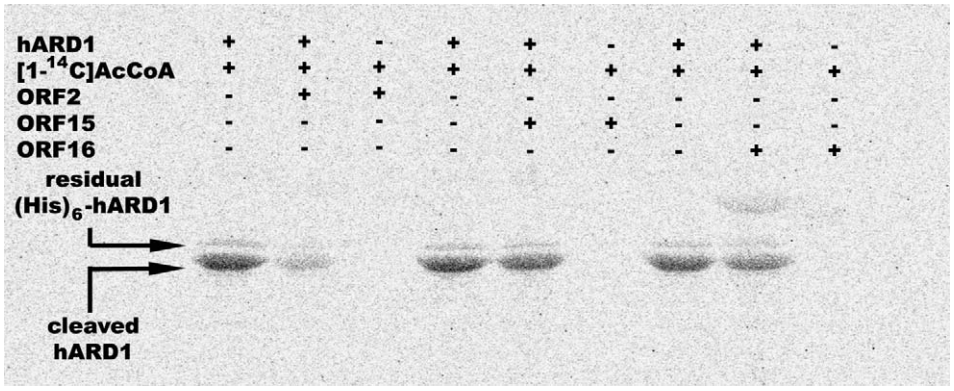

Fig. 5. Autoradiograph showing incubations of cleaved hARD1 (lanes 1–2, 4–5 and 7–8) with [1-^{14}C]-AcCoA and ORFs 2, 15 and 16 (lanes 2–3, 5–6 and 8–9) from the clavulanic acid biosynthesis pathway as control proteins with glycyl residues at their *N*-termini. A faint band corresponding to residual $(His)_6$-tagged hARD1 is visible above the cleaved hARD1.

residues as substrates for Nα-acetylation, and suggest that, whilst it cannot be ruled out, there is no evidence that N-terminal acetylation of hARD1 occurs in human cells [19]. Given that N-terminal Met, Asn or Val (as in some homologues) residues are not reported to be substrates for the NatA complex, (for review, see [26]) it is possible that wild-type hARD1 and

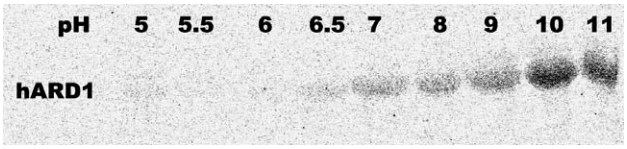

Fig. 6. [^{14}C]-Autoradiograph of incubations of $(His)_6$-tagged hARD1 with [1-^{14}C]-AcCoA at different pH values in the range 5–11.

homologues have an N-terminal sequence that has evolved to be refractory to self-*N*-acetylation.

Although Jeong et al. [15] reported that addition of NATH (also known as Tubedown-100) to murine ARD1 did not affect the acetylation of HIF-1α, a complex between hARD1 and NATH has been observed and found to possess N-terminal acetyltransferase activity with peptide substrates, consistent with observations for the yeast enzyme [18]. Thus, Arnesen et al. noted that mammalian ARD1 appears to be a novel acetyltransferase that was capable of both Nα- and Nε-protein acetylation, in complex with NAT and alone, respectively. Taken together with other recent studies on cells from Bilton et al. [21] and Fisher et al. [20] the work described here with purified proteins implies that hARD1 does not, at least alone, catalyse the acetylation of Lys_{532} of human HIF-1α and as a consequence is likely not directly involved in its regulation, at least via a Lys_{532} acetylation-mediated process. The available evidence indicated that hARD1 is in fact a more typical component of a heterodimeric complex with NATH that catalyses N-terminal acetylation reactions. The mechanism by which this, or other activity, of hARD1 affects levels of the biomedicinally important proteins EPO and VEGF is therefore unclear, and is a direction for further study of hARD1.

Note added in proof

Recently, Arnesen et al. [27] have reported evidence that the stability of HIF-1α is not regulated by hARD1 and that the level of hARD1 is not hypoxically regulated. These authors also reported evidence that hARD1 does not acetylate HIF-1α, but importantly did demonstrate an interaction between hARD1 and HIF-1α.

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